IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Berish Rubin, et al.

Serial No: 10/050,189

Filed: January 16, 2002

For: DETECTION OF MUTATIONS IN
A GENE ENCODING IKAPPAB
KINASE-COMPLEX-ASSOCIATED
PROTEIN TO DIAGNOSE
FAMILIAL DYSAUTONOMIA

Examiner: Carla J. Myers

Art Unit: 1634

Docket: 18090

Confirmation No.: 6280

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

DECLARATION PURSUANT TO 37 C.F.R. §1.131

Sir:

We, Berish Y. Rubin and Sylvia L. Anderson, hereby declare that:

1. We are co-inventors named in the above-identified application.

2. We have been advised by our legal representative that published U.S. Application 2002/0169299 (" the '299 application"), filed by Slaugenhaupt et al., has been cited by the Examiner as a prior art reference in respect to the above-identified application. The '299 application claims priority from U.S. Provisional Application No. 60/260,080, filed on January 6, 2001.

3. The presently claimed invention was conceived and reduced to practice in the United States prior to January 6, 2001, the priority date of the '299 application.

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4.   We provide herewith **Exhibits 1-4** as evidence of conception and reduction to practice of the present invention prior to January 6, 2001. Dates have been masked in the preparation of the photocopies contained in Exhibits A and B.

5.   **Exhibit 1** is a copy of an email dated prior to January 6, 2001, from the Editorial Coordinator of the journal Science, Carolyn Kyle, to Dr. Berish Rubin, acknowledging the submission of a manuscript from Dr. Rubin. As indicated in the email, the manuscript is entitled "Familial Dysautonomia is Caused by Mutations of the IKAP Gene."

6.   **Exhibit 2** is a copy of an email dated prior to January 6, 2001, from Dr. Rubin to Mr. Kurt G. Briscoe, a patent attorney at Norris McLaughlin & Marcus, P.A., 220 East 42nd Street, New York, New York 10017. In the email, Dr. Rubin indicated that a manuscript was attached and requested that Mr. Briscoe prepare and file a patent application based on the manuscript.

7.   **Exhibit 3** is a copy of an email dated prior to January 6, 2001, from Mr. Kurt G. Briscoe to Dr. Rubin, attaching "a first draft of the application".

8.   **Exhibit 4** is a copy of the first draft of the application, which was attached to the email of **Exhibit 3**.

9.   The content of the first draft application in **Exhibit 4** substantially corresponds to the present application. In particular, Figures 1-5 of the first draft application are identical to Figures 1-5 of the present application. Claims 1-8 of the first
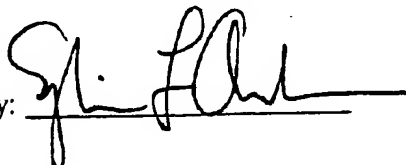
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draft application substantially correspond to original claims 1-8 of the present application. The subject matter of claims 14-17 of the present application is also disclosed in the first draft application, e.g., the paragraph bridging pages 4-5 of the present application and Figure 1.

10.   Therefore, the subject matter claimed in the present application was conceived and reduced to practice prior to January 6, 2001. A U.S. provisional application (No. 60/262,284) was diligently filed on January 17, 2001, which was converted to the present utility application on January 16, 2002.

11.   We declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and that those statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

By: _____   Dated: ___5/4/2005___

By: _____   Dated: ___5-4-05___

Manuscript Title:"Familial Dysautonomia is Caused by Mutations of the IKAP
Gene"

Manuscript Number:1057602

Dear Dr. Berish Rubin

 Thank you for submitting your manuscript to Science. We will notify you of
our evaluation as soon as possible. Your manuscript number is noted above.
Please refer to it in future communication with us.

Sincerely,

Carolyn Kyle
Editorial Coordinator
202-326-6550

Berish Rubin /FACULTY/FIRE   ·· To kgbriscoe@nmmlaw.com

cc sanderson@fordham.edu

bcc FRANCES J. NAVARRO/STAFF/FIRE@FIRE

Subject Manuscript

Dear Kurt,
After a great deal of effort, we have completed and submitted a manuscript describing our findings. I am attaching the manuscript and hope that with this manuscript and the information we sent you earlier you have sufficient information to file the patent.

Thanks, Berish andersontextfigs.doc

Dear Dr. Rubin,

First, please let me know that you received this email.

Second, attached is a first draft of the application, along with a commentary that includes numerous questions. Please review both, and let me have your comments and answers.

Best regards and Happy Holidays.

Kurt(See attached file: RUBIN201.FIRST-DRAFT.doc)(See attached file: RUBIN201.COMMENT.doc)

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DETECTION OF MUTATIONS IN A GENE ENCODING
IκB KINASE-COMPLEX-ASSOCIATED PROTEIN
TO DIAGNOSE FAMILIAL DYSAUTONOMIA

This invention relates to a method for the diagnosis of Familial Dysautonomia, and to a mutated gene and altered proteins associated therewith.

Familial Dysautonomia (FD), also known as the Riley-Day syndrome or hereditary sensory neuropathy Type III (MIM 223900), is an autosomal recessive disorder that affects the development and survival of sensory, sympathetic and some parasympathetic neurons (*1,2*). Individuals with FD are affected with a variety of symptoms that include decreased sensitivity to pain and temperature, cardiovascular instability, recurrent pneumonias, vomiting crises, and gastrointestinal dysfunction (*1-3*). It has been suggested that these symptoms might be due to a deficiency in a neuronal growth factor pathway (*4,5*). This disorder is primarily confined to individuals of Ashkenazic Jewish descent (*6*). Based on the birth incidence of FD, the predicted carrier frequency of this defective gene (*DYS*) is approximately 1 in 30 (*7*). Haplotype analysis using various polymorphic loci has enabled the localization of FD to chromosome 9q31 and the demonstration that there is a major haplotype representing >98% of the FD chromosomes (*8*). Several additional FD haplotypes represent the remaining 2% of FD chromosomes. The

defective gene, *DYS*, which has been mapped to a 0.5 cM region on chromosome 9q31, has eluded identification.

Identification of the gene and/or altered protein associated with FD would be valuable, as knowledge of such gene and/or altered protein would allow more accurate screening of the entire at-risk population to identify carriers, and potentially to reduce the incidence of new cases. Thus, genetic testing would permit the identification of carriers of a disease gene, and also the evaluation of the condition of unborn children who may be affected with the disease or carriers of the disease gene. Knowledge of the gene and/or altered protein associated with FD might also facilitate the development of effective therapeutic approaches for individuals with FD.

With the release of the DNA sequence of the human genome, we determined the location of the polymorphic markers 164D1, D9S1677 and 157A3 that have been reported to be localized to the area of the *DYS* gene (*8*) and identified seven mRNAs and a number of ESTs (expressed sequence tags) encoded in this region. RT-PCR analysis performed on these RNAs revealed that primers to the transcript reported to encode the IκB kinase-complex-associated protein (IKAP) (*9*), which generated the predicted 218 bp product from RNA isolated from a control lymphoblast cell line, generated a 144 bp PCR product from RNA derived from a lymphoblast cell line generated from an individual homozygous for the major FD haplotype. The same primers generated both the 218 and 144 bp products from RNA isolated from lymphoblast cell lines derived from individuals heterozygous for the major FD haplotype (Fig. 1A). Identical

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results were obtained when using RNAs from fibroblast cell lines derived from normal

individuals and individuals homozygous and heterozygous for *DYS* (data not shown). Sequence

analysis of the RT-PCR products revealed that the IKAP mRNA generated by the *DYS*-bearing

chromosome does not contain exon 20 and that the predicted translation of this transcript

generates a truncated protein with a molecular weight of approximately 79 kD (Fig. 1B).

Western blot analysis using antibody to IKAP reveals the presence of a 150 kD protein in control

cells and a 79 kD protein in cells derived from individuals homozygous for the major FD

haplotype (Fig. 1C).

Using DNA sequence information available as a result of the Human Genome Project,

we characterized the RNAs encoded by this region of chromosome 9 and demonstrate that

mutations in the gene encoding the protein termed IκB kinase-complex-associated protein

(IKAP) are responsible for FD.

Accordingly, the present invention relates in a first aspect to the identification of the gene

responsible for FD, and the pertinent mutations and altered proteins associated therewith.

The present invention relates in a second aspect to a method for detecting the presence in

a subject of a polymorphism linked to a gene associated with familial dysautonomia, said method

comprising detecting a disruptive mutation in a gene of said subject encoding the IκB kinase-

complex-associated protein.

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The present invention relates in a third aspect to novel primers useful in the diagnostic methods according to the teachings of the present invention.

The accompanying drawings, which illustrate various aspects of the present invention, can be summarized as follows:

Fig. 1. A deletion in IKAP mRNA results in a truncated protein. **(A)** RT-PCR analysis of IKAP RNA. RT-PCR analysis was performed on RNA isolated from lymphoblast cell lines generated from individuals lacking the *DYS* allele (normal), or heterozygous (carrier) or homozygous (affected) for the major *DYS* allele. DNase-treated total RNA was amplified with primers spanning exons 19-21 (5'-GCAGCAATCATGTGTCCCA-3' and 5'-GATTCTCAGCTTTCTCATGC-3'). Arrows indicate the positions of the DNA markers run on the gel. **(B)** Alignment of normal and FD IKAP cDNA and amino acid sequences. An alignment of a portion of exons 19-21 of normal IKAP cDNA with that of IKAP cDNA from an FD-affected individual demonstrating that the exclusion of exon 20 in the RNA transcribed from the FD allele results in a frame shift, causing premature termination of translation and a protein truncated by 619 amino acids. Normal IKAP has 1332 amino acids. **(C)** Western blot analysis of IKAP protein in a non-FD lymphoblast cell line (DY491) and a lymphoblast cell line established from an FD-affected individual (GM05106). The blot was probed with polyclonal antibody to IKAP prepared in goat against the 18 aa peptide, DPVSREVKNEVSLVAEGF, encoded in exons

2 and 3. The presence of IKAP was detected with an anti-goat antibody conjugated to alkaline

phosphatase. A prestained molecular weight marker was used to determine sizes of products

(not shown).

Fig. 2. T→C transition in a donor splice site results in excision of exon 20. **(A)** Sequence

analysis of IKAP intron 20 donor splice sites in normal and FD alleles. PCR fragments,

amplified from DNA derived from normal cells and cells homozygous for the major FD

haplotype, spanning the intron 20 donor splice of IKAP were sequenced. The normal donor

splice sequence is GTAAGTG, the FD sequence is GTAAGCG. **(B)** Splicing of the IKAP

transcript. Normal splicing of the IKAP transcript results in removal of introns 19 and 20 and

retention of exon 20. The T→C transition in the donor splice site of intron 20 in the mutant

allele results in removal of introns 19 and 20, as well as exon 20. The base change in the donor

splice site is underlined.

Fig. 3. A G→C transversion prevents phosphorylation. **(A)** Sequence analysis of exon 19

of IKAP cDNA. cDNA from a normal individual and a carrier of the minor 2 haplotype was

sequenced from PCR fragments spanning exon 19. **(B)** Immunoprecipitation of ^{35}S-methionine

labeled versus ^{32}P-orthophosphate labeled IKAP. DY491, a normal lymphoblast cell line, and

DY374, a lymphoblast cell line established from a carrier of the minor 2 FD haplotype, were

labeled for six hours with ^{35}S-methionine or ^{32}P-orthophosphate. Whole cell extracts were

prepared from these cells and equal amounts of acid-precipitable radioactivity were

immunoprecipitated with an IKAP polyclonal antibody (described in Fig. 1C). The levels of

radiolabeled IKAP protein precipitated from ^{35}S-methionine and ^{32}P-orthophosphate labeled

DY374 cells, respectively, are depicted, in the bar graph, as a percentage of the radiolabeled

protein precipitated from DY491 cells. The standard deviation for four experiments is indicated.

Above the bars is an autoradiograph depicting the immunoprecipitated ^{35}S –labeled IKAP from

DY491 (a) and DY374 (b) cells and the immunoprecipitated ^{32}P-labeled IKAP from DY491 (c)

and DY374 (d) cells. Immunoprecipitations of IKAP from three other non-FD cell lines yielded

results similar to that observed with the DY491 cell line (data not shown).

Fig. 4. Genotype analysis, using SSCP, of FD alleles. **(A)** PCR of the FD major allele in

an extended family. A 244 bp fragment was amplified from DNA purified from blood using

primers 5′-GAGAACAACAAGATTCTGC-3′ and 5′-AGTCGCAAACAGTACAATGG-3′ in

the presence of α-^{33}P-dATP. The amplified products were denatured and fractionated on a 5%

acrylamide non-denaturing gel at 4°C. Open circles (females) or squares (males) represent

carriers of normal alleles, circles or squares with black circles inside represent heterozygous

carriers of the major FD haplotype and filled circles and squares, FD-affected homozygous

individuals with two mutated alleles. **(B)** Multiplex PCR of FD major and FD minor 2 alleles in

two families. PCR was performed as in panel A, except two additional primers, 5′-

GCAGTTAATGGAGAGTGGCT-3′ and 5′-ATGCTTGGTACTTGGCTG-3′, which generate a

238 bp DNA fragment, were included in the reactions. PCR reactions were analyzed as above.

Parental carriers of the major or minor 2 allele are shown as circles (female) or squares (male)

with horizontal lines or vertical lines, respectively. FD-affected offspring with both major and

minor 2 alleles are shown as crosshatched.

Fig. 5. Differential expression of IKAP by tissue type. A Multiple Tissue

Expression Array (Clontech) containing RNA from 76 different human tissues and

developmental stages was probed with a radiolabeled 556 bp cDNA fragment spanning exons

23-27 of IKAP. The probed array was subjected to autoradiography and densitometric scanning

to quantitate relative levels of tissue expression. The 20 tissues that showed the highest level of

expression are depicted. The highest level of expression was observed in the cerebellum, whose

level was set at 1.0; the relative expression levels in the other 19 tissues are shown. The amounts

of poly A+ RNA in the tissue samples on the array have been normalized based on eight

housekeeping genes.

Sequence analysis of the IKAP-encoding gene reveals, in chromosomes with the major

FD haplotype, a T → C transition in position 6 of the donor splice site of intron 20 (Fig. 2A).

This mutation (2507+6T→C) results in the generation of an IKAP mRNA in which exon 20 is

spliced out along with intron 20 (Fig. 2B).

Characterization of the IKAP-encoding gene of individuals heterozygous for the FD

chromosome with the most common minor haplotype (minor 2) (*8*) reveals, in this allele, a G →

C transversion of nucleotide 2390 in exon 19 of the reported IKAP cDNA (*9*; Genbank

accession #NM_003640), resulting in an arginine → proline substitution of amino acid residue 696 of IKAP (R696P) (Fig. 3A) and the disruption of a consensus serine/threonine kinase phosphorylation site (RIVT→pIVT). Thus, probands with both the 2507+6T→C and R696P mutations produce a truncated IKAP as well as a full-length IKAP with a non-consensus phosphorylation site, respectively.

Immunoprecipitation of IKAP from ^{35}S-methionine or ^{32}P-orthophosphate-labeled cells derived from a normal individual and an individual heterozygous for R696P revealed comparable levels of synthesis of IKAP but a reduced level of phosphorylation of this protein in cells bearing the R696P mutation (Fig 3B).

SSCP analysis capable of differentiating between the normal and the mutated sequences of IKAP can be used to diagnose FD. SSCP analysis was performed on a multigenerational family with several FD-affected individuals bearing the major FD haplotype (Fig. 4A) and two pairs of apparently unrelated parents with probands that have alleles corresponding to both the major and minor 2 FD haplotypes (Fig. 4B). In the family with probands homozygous for the major haplotype, all of the affected individuals were homoallelic for 2507+6T→C and all of the parents were heterozygous. In the families with probands heterozygous for the major and minor 2 FD haplotypes, for both pairs of parents, one parent and the proband are heterozygous for the R696P and the other parent and the proband are heterozygous for 2507+6T→C (Fig. 4B).

Analysis of 31 probands homozygous for the major FD haplotype revealed that 100% of the probands were homozygous for 2507+6T→C, 100% of the parents were heterozygous for this mutation and four siblings of the probands had FD and were homozygous for the FD haplotype and the 2507+6T→C mutation. No other families with probands with the minor 2 FD haplotype were available for analysis. Study of a random group of 619 individuals of Ashkenazic Jewish descent revealed the presence of 25 carriers of 2507+6T→C and two individuals with R696P. This observed FD carrier frequency of approximately 1 in 23 is slightly higher than the reported frequency (7) and may reflect either an under-estimation based on the birth frequency or the nature or size of the sample characterized in this study. Each of the individuals with the 2507+6T→C and R696P mutations was found to have the polymorphic DNA markers associated with the FD major and minor haplotypes, respectively (8).

Characterization of the expression of the IKAP mRNA in multiple tissues revealed the greatest level of expression in the cerebellum, thalamus, pituitary and testes and significant expression in several regions of the brain (Fig. 5). Little IKAP mRNA was detected in colon, lung and ovary (data not shown). Many of the neurologic disorders observed in FD-affected individuals have been attributed to incomplete development of sensory and autonomic neurons (1-3). The high level of expression of IKAP mRNA in nervous tissues and the defective synthesis of this protein in individuals with FD suggest that the absence of normal IKAP may play a role in the observed abnormal intrauterine development and postnatal maintenance of neurons (3). Furthermore, the abundant expression of IKAP mRNA in the cerebellum and

thalamus suggest that the disturbances of gait and coordination, as well as inappropriate perception of pain and temperature, might be due in part to dysfunctions in these brain loci, respectively.

IKAP was initially identified and named based on its reported ability to bind the IκB kinases (IKKs), the NF-κB inhibitory subunit IκB-α, NF-κB and the NF-κB-inducing kinase (NIK) and assemble these proteins into an active kinase complex (9). Recent studies, however, suggest that IKAP is not associated with the IKKs and plays no specific role in cytokine-induced NF-κB activation (10). Characterization of the amino acid sequence of IKAP reveals significant amino acid sequence homology with the *Saccharomyces cerevisiae* IKI3 (11) and ELP1 (12) proteins as well as similar proteins in *Schizosaccharomyces pombe* and *Arabidopsis thaliana*. The IKI3 gene product mediates, by a yet to be determined mechanism, sensitivity to the yeast killer toxin (11). ELP1 is a subunit of a multisubunit complex that is associated with RNA polymerase II and is required for the activation and transcriptional elongation of a large number of genes (12). If IKAP, like ELP1, is a part of the RNA polymerase II elongation complex and plays a role in gene activation, the absence of functional IKAP in FD-affected individuals may prevent gene activation events necessary for normal neuronal development and function.

Identification of the mutations responsible for FD will enable the identification of carriers of this genetic disorder and may result in the development of effective therapeutic approaches for individuals with FD.

It should be understood that the preceding is merely a detailed description of one embodiment of this invention and that numerous changes to the disclosed embodiment can be made in accordance with the disclosure herein without departing from the spirit or scope of the invention. The preceding description, therefore, is not meant to limit the scope of the invention. Rather, the scope of the invention is to be determined only by the appended claims and their equivalents.

References Cited

The references cited in the foregoing description are as follows:

1. C. M. Riley, R. L. Day, D. Greely, W. S. Langford, *Pediatrics* **3**, 468 (1949).

2. F. B. Axelrod, R. Nachtigal, J. Dancis, *Adv. Pediatr.* **21**, 75 (1974).

3. F.B. Axelrod, in *Primer on the autonomic nervous system*, D. Robertson, P. A. Low, R. J. Polinsky, Eds. (Academic Press, San Diego, 1996), pp. 242-249.

4. X. O. Breakefield *et al.*, *Proc. Natl. Acad. Sci.* **81**, 4213 (1984).

5. X. O. Breakefield *et al.*, *Mol. Biol. Med.* **3**, 483 (1986).

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7. C. Maayan, E. Kaplan, S. Shachar, O. Peleg, S. Godfrey, *Clin. Genet.* **32**, 106 (1987).

8. A. Blumenfeld *et al.*, *Am. J. Hum. Genet.* **64**, 1110 (1999).

9. L. Cohen, W. J. Henzel, P. A. Baeuerle, *Nature* **395**, 292 (1998).

10. D. Krappmann *et al.*, *J. Biol. Chem.* **275**, 29779 (2000).

11. H. Yajima, M. Tokunaga, A. Nakayama-Murayama, F. Hishinuma, *Biosci. Biotechnol. Biochem.* **61**, 704 (1997).

12. G. Otero *et al.*, *Mol. Cell.* **3**, 109 (1999).

WHAT IS CLAIMED IS:

1. A method for detecting the presence in a subject of a polymorphism linked to a gene associated with familial dysautonomia, said method comprising detecting a disruptive mutation in a gene of said subject encoding the IκB kinase-complex-associated protein.

2. The method according to claim 1, which comprises detecting a disruptive mutation in the gene encoding the IκB kinase-complex-associated protein which is present on chromosome 9q31.

3. The method according to claim 2, which comprises detecting a T → C change in position 6 of the donor splice site of intron 20 of the gene encoding the IκB kinase-complex-associated protein which is present on chromosome 9q31.

4. The method according to claim 2, which comprises detecting a G → C transversion of nucleotide 2390 in exon 19 of the gene encoding the IκB kinase-complex-associated protein which is present on chromosome 9q31.

5. The method according to claim 3 or 4, which comprises detecting said T → C

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change and/or said G → C transversion by single-stranded conformation

polymorphism (SSCP) analysis.

6.   The method according to claim 5, wherein said SSCP analysis is carried out on a

nucleic acid sequence amplified by polymerase chain reaction (PCR).

7.   The method according to claim 6, wherein said nucleic acid sequence is amplified

by PCR using one or more oligonucleotide primers selected from the group

consisting of:

a)   GAGAACAACAAGATTCTGC;

b)   AGTCGCAAACAGTACAATGG;

c)   GCAGTTAATGGAGAGTGGCT; and

d)   ATGCTTGGTACTTGGCTG.

8.   An oligonucleotide primer selected from the group consisting of:

a)   GAGAACAACAAGATTCTGC;

b)   AGTCGCAAACAGTACAATGG;

c)   GCAGTTAATGGAGAGTGGCT; and

d)   ATGCTTGGTACTTGGCTG.

ABSTRACT OF THE DISCLOSURE

A method for detecting the presence in a subject of a polymorphism linked to a gene associated with familial dysautonomia, said method comprising detecting a disruptive mutation in a gene of said subject encoding the IκB kinase-complex-associated protein, and, preferably, detecting a T → C change in position 6 of the donor splice site of intron 20 and/or a G → C transversion of nucleotide 2390 in exon 19 of the gene encoding the IκB kinase-complex-associated protein which is present on chromosome 9q31. Also disclosed are oligonucleotide primers useful in the detection method. This abstract is provided to comply with the rules requiring an abstract that will allow a searcher or other reader to ascertain quickly the subject matter of the technical disclosure. It is submitted with the understanding that it will not be used to interpret or limit the scope or meaning of the claims. 37 CFR § 1.72(b).

A

Fig. 1

bp

Normal Carrier Carrier Carrier Affected

300 →

200 →

100 →
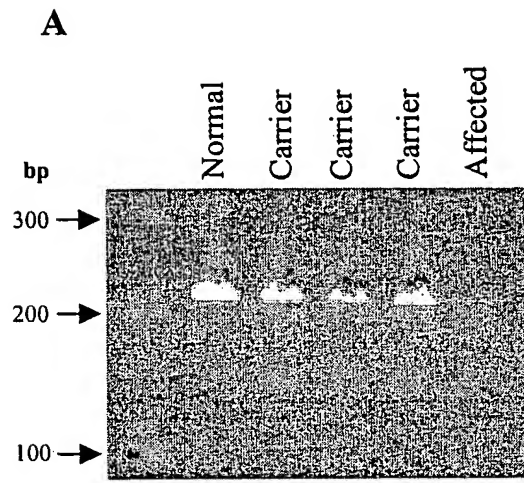
B

<u>Alleles</u>

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        P  Q  D  T  K  L  V  L  Q  M  P  R  G
Normal  CCCCAGGACACAAAGCTTGTATTACAGATGCCAAGGGGA
FD      CCCCAGGACACAAAGCTTGTATTACAG------------
        P  Q  D  T  K  L  V  L  Q

        N  L  E  V  V  H  H  R  A  L  V  L  A
Normal  AACTTAGAAGTTGTTCATCATCGAGCCCTGGTTTTAGCT
FD      ---------------------------------------

        Q  I  R  K  W  L  D  K  L  M  F  K  E
Normal  CAGATTCGGAAGTGGTTGGACAAACTTATGTTTAAAGAG
FD      ----------------------ACTTATGTTTAAAGAG
                              T  Y  V  *
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C

Normal Affected

150kD →

79kD

17

A

Fig. 2

Normal   Affected

B

Normal

Exon 19   Exon 20   Exon 21

gtaagt

FD

Exon 19   Exon 20   Exon 21

gtaagc

Fig. 3

A

Normal   Heteroalleli

B

Fig. 4

A

B

Mutated major allele

Mutated minor allele

Mutated major allele

Fig. 5